

## Electron-Spin-Resonance Evidence for Enzymic Reduction of Oxygen to a Free Radical, the Superoxide Ion

BY P. F. KNOWLES, J. F. GIBSON, F. M. PICK AND R. C. BRAY

Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, London, S.W. 3,  
and Department of Chemistry, Imperial College, London, S.W. 7

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1. An electron-spin-resonance signal with  $g_{\parallel}$  2.08 and  $g_{\perp}$  2.00 is observed by the rapid-freezing technique during the oxidation of substrates by molecular oxygen catalysed by xanthine oxidase at pH10. 2. The intensity of this signal is shown to depend on oxygen rather than on enzyme concentration, indicating that it is due to an oxygen free radical and not to the enzyme. 3. The same species is shown to be produced in the reaction at pH10 between hydrogen peroxide and periodate ions. Studies with this system have facilitated comparison of the properties of the oxygen radical with data in the literature on the products of pulse radiolysis of oxygenated water over a wide pH range. 4. It is concluded that the species observed is the superoxide ion,  $O_2^-$ , and that the stability of this ion is greatly increased in alkaline solution. A mechanism explaining the alkaline stability is proposed. 5. The importance of  $O_2^-$  in the enzymic reaction is discussed.

Enzymic reduction of molecular oxygen to hydrogen peroxide may in principle occur as two single-electron steps or as one two-electron step. Although there is considerable indirect evidence that the two-step mechanism operates in certain enzyme reactions (Mason, 1965) we are not aware that formation of an oxygen radical intermediate has ever been demonstrated entirely unequivocally. Electron-spin resonance (ESR) provides a powerful and unambiguous technique for searching for such a species and we now present evidence that an ESR signal observed at pH10 during the oxidation of substrates by oxygen catalysed by xanthine oxidase is due to  $O_2^-$ , the superoxide free radical anion (superoxide is the name recommended by The Chemical Society for  $O_2^-$ ; other names are hyperoxide, hydroperoxy and perhydroxyl), stabilized by the alkaline medium, but not interacting with the enzyme molecule. The enzymically produced radical is identical with one obtained by oxidation of hydrogen peroxide by periodate ions and by other means. ESR parameters of the signal are compared with values in the literature for  $O_2^-$  and the stability of this ion is discussed. A preliminary report on this work has been published (Bray, Knowles, Pick & Gibson, 1968).

### MATERIALS AND METHODS

Xanthine oxidase was prepared by the method of Hart & Bray (1967). The rapid-freezing technique of Bray (1961) with later modifications (Bray, Knowles & Meriwether,

1967) was used to obtain samples frozen after short reaction periods for ESR measurement. In agreement with the fact that a rather labile species (Bray & Knowles, 1968) was being investigated, it was found that larger radical signals were obtained when smaller jets were employed (Palmer, Bray & Beinert, 1964). ESR measurements were carried out on a Varian V-4502 X-band spectrometer with dual-sample cavity and variable-temperature accessory. Measurements of  $g$  values (to  $\pm 0.002$ ) and absolute signal-intensity measurements were carried out as described by Bray *et al.* (1967).

### RESULTS

*Oxygen radical ESR signal produced by xanthine oxidase.* Fig. 1(a) shows ESR signals obtained during the reaction of oxygenated xanthine solutions with xanthine oxidase at pH10. Results are in full agreement with those of Bray, Palmer & Beinert (1964). Signals due to Mo(V) in the reduced enzyme are observed, together with an asymmetric radical signal in the region of  $g=2.00$ . As previously reported (Bray *et al.* 1964), the latter signal was apparent only at intermediate reaction times and was considerably stronger at pH10 than at pH 8.2. A more detailed investigation of the signal at pH10 revealed (Bray & Knowles, 1968) that it has an additional weak component in the region of  $g=2.08$  (the low broad hump towards the left in Fig. 1a) and that it therefore arises from a species having axial symmetry. Our parameters for the signal are  $g_{\parallel}$  2.081 and  $g_{\perp}$  2.001 [ $g_{\perp}$  is here taken as the point of maximum slope on the absorption

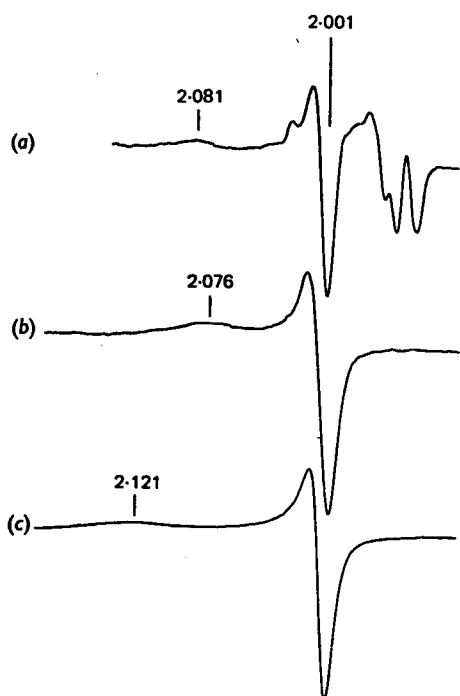


Fig. 1. ESR signals ascribed to  $O_2^-$  and obtained both enzymically and non-enzymically; the rapid-freezing technique (Bray, 1961) was used for preparing the samples and spectra were recorded at  $-170^\circ$ . (a) Reaction between xanthine (2.0 mM) and  $O_2$  (0.76 mM) catalysed by xanthine oxidase (0.20 mM) at pH 10.0 and  $23^\circ$ , after a reaction time of 150 msec. Molybdenum signals (overmodulated) are also present, and an iron signal is responsible for the sloping base line. (b) Reaction between  $H_2O_2$  (140 mM) and  $NaIO_4$  (70 mM) at pH 9.9 (carbonate buffer) after a reaction time of 600 msec. (c) As for (b) but at pH 13.2 (KOH). For both (b) and (c) a three-syringe system (Palmer *et al.* 1964) was employed, to enable the peroxide to be made alkaline only shortly before the addition of periodate.

curve (Symons, 1963), though in an earlier report (Bray & Knowles, 1968) we used  $g_m$ , the point of maximum absorption]. The  $g_1$  component of the signal sharpens considerably on cooling from  $-150^\circ$  to  $-180^\circ$ , but this component is clearly distinguished from non-haem iron signals from the enzyme by the fact that it saturates more readily on increasing the microwave power. Results of an investigation of oxygen concentration and of enzyme concentration on the intensity of the radical and other signals produced by the enzyme system are presented in Table 1. The relative intensity of the radical clearly is the same whether it is measured at the  $g_{\perp}$  or the  $g_1$  component, confirming that a single species is responsible. On decreasing the oxygen concentration the radical signal decreases and it approaches zero intensity when anaerobic conditions are employed. When the enzyme concentration was decreased from 0.198 mM to 0.062 mM, i.e. to 0.31 times the original value, the intensity of the radical signal did not decrease proportionately. Indeed under some conditions it actually increased in intensity in this experiment. In contrast, the molybdenum and iron signals were relatively insensitive to the presence or absence of oxygen and in every case these signals were much weaker with the dilute than with the concentrated enzyme. Combining the data for the two molybdenum signals and the iron signal at the three oxygen concentrations in Table 1, the average change in signal intensity was to 0.39 times the original value when the enzyme concentration was decreased to 0.31 times the original value.

The data thus provide strong evidence that the radical signal is associated with oxygen rather than with the enzyme. Integration of the oxygen radical signal produced in experiments of this type at pH 10 showed that radical concentrations up to about 0.06 mM could be attained by using a starting oxygen concentration of 0.76 mM.

Table 1. Intensities of ESR signals during the oxidation of xanthine at pH 10: effect of varying the xanthine oxidase and oxygen concentrations

Enzyme concentrations of 0.062 mM ('Dil.') and 0.198 mM ('Conc.') were employed. Each signal was measured under each set of conditions at reaction times of 25, 145, 310 and 425 msec. at  $23^\circ$  by using the rapid-freezing technique (Bray, 1961). The maximum intensities attained within this time range are those recorded. For each species the intensity of the strongest signal in the whole series was taken as 100. The signal ascribed to  $O_2^-$  was measured both at  $g_{\perp}$  (2.001) and at  $g_1$  (2.081) (see Fig. 1a); molybdenum and iron signals were measured as described by Palmer *et al.* (1964).

Concn. of $O_2$ (mM)	Maximum height of signal (relative)									
	$O_2^- (g_{\perp})$		$O_2^- (g_1)$		Mo ( $\delta$ peak)		Mo ( $\beta$ peak)		Fe	
	Dil.	Conc.	Dil.	Conc.	Dil.	Conc.	Dil.	Conc.	Dil.	Conc.
0.76	76	100	78	100	21	100	15	85	22	97
0.26	52	19	50	28	24	71	27	100	39	97
0	10	10	0	11	15	47	38	42	72	100

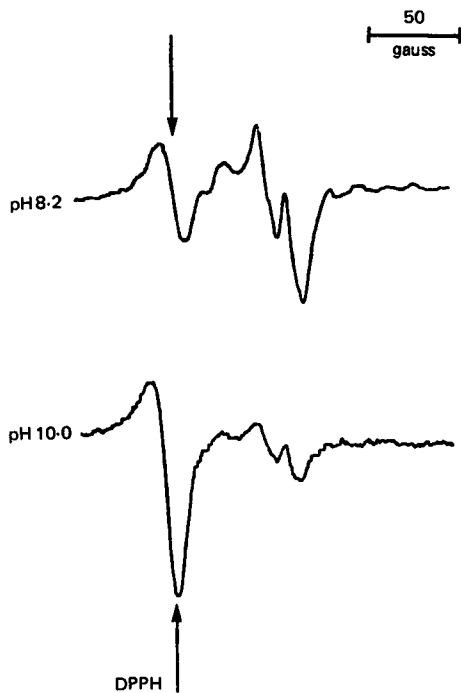


Fig. 2. ESR signals obtained during reaction between salicylaldehyde (20mM) and  $O_2$  (0.26mM) catalysed by xanthine oxidase (approx. 0.1mM) at pH 8.2 and pH 10.0. Reaction times were approx. 450msec. At the higher pH the signal in the region of the diphenylpicrylhydrazyl (DPPH) marker is ascribed to the  $g_{\perp}$  component of  $O_2^-$ ; at the lower pH the weaker, more symmetrical, signal in the same region is probably due to FADH.

It was confirmed that the radical could be produced from oxygen by xanthine oxidase utilizing substrates other than xanthine. Fig. 2 shows that the form of the signal is unchanged when salicylaldehyde is used at pH 10. However, with this substrate at pH 8.2 the signal in the  $g = 2.00$  region was symmetrical and is therefore clearly due to a species different from that arising at the higher pH.

*Oxygen radical ESR signal produced by an inorganic system.* A non-enzymic system producing oxygen radicals was sought in which their ESR signals could be studied over a wide range of pH and was found in the reaction between hydrogen peroxide and periodate. Although oxygen radicals do not seem previously to have been implicated in this reaction, it does have features (Anbar, 1961) in common with well-known  $HO_2$ -producing systems (Barb, Baxendale, George & Hargrave, 1951). At pH 10, on mixing hydrogen peroxide with periodate, signals almost indistinguishable in form and in  $g$  values from those generated in the enzyme system were produced (Fig. 1b). The observed  $g$  values

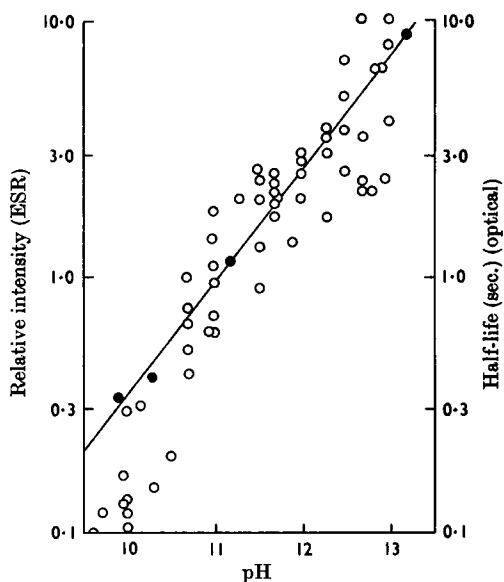


Fig. 3. Effect of pH on the intensity of the ESR signal ascribed to  $O_2^-$  produced on treating  $NaIO_4$  with  $H_2O_2$ . Reaction conditions were as in Figs. 1(b) and 1(c). The amplitude of the signal was measured at  $g_{\perp}$  (●) and full line). For comparison, data replotted from Czapski & Dorfman (1964) on the half-life of the 'alkali stabilized form of  $O_2^-$ ', obtained by pulse radiolysis of oxygenated water and detected by the extinction at  $240m\mu$  are also shown (○).

were:  $g_{\parallel} 2.076$ ;  $g_{\perp} 2.001$ . As a further test for the identity of the species, the stability of the signals on warming up the frozen matrices was studied (Bray & Knowles, 1968) and rather similar stabilities were found. Thus at  $-65^\circ$  the signal, obtained at pH 10 either with the enzyme or with the hydrogen peroxide-periodate system, disappeared gradually. Though the fading rate was somewhat greater in the former case, this could well have been due to continuing radical generation in the inorganic system, rather than to genuine radical stability differences. It is concluded that the same species is being generated in the inorganic as in the enzymic system and that the very minor differences in the  $g$  values are to be attributed to slight differences in the media (see the Discussion section).

Further, less complete, experiments demonstrated that the same oxygen radical signal could be detected during a number of other inorganic reactions such as in hydrogen peroxide oxidation by hypochlorite or in dithionite reduction of oxygen. However, in the latter case the situation appeared to be complicated by the presence additionally of the  $SO_2^-$  radical (Atkins, Horsfield & Symons, 1964).

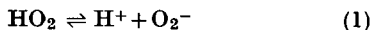
The yield of the oxygen radical produced by the

hydrogen peroxide-periodate system and the parameters of the signal have been studied as a function of pH in the range 2.8–13.2. In the neutral region signals were very weak, and at the lowest pH value a rather weak signal resembling that ascribed to  $\text{HO}_2$  by Ichikawa, Iwasaki & Kuwata (1966) was observed. In the alkaline region the yield of the radical increased rapidly with increasing pH (Fig. 3). There were also some changes of  $g$  values in the alkaline region, the values observed at pH 13.2 being:  $g_{\parallel}$  2.121;  $g_{\perp}$  2.001 (Fig. 1c). At the highest pH values, signal intensities corresponded to concentrations of the radical of about 2 mM. The effect of pH on the yield of our species is strikingly similar to the effect of pH on the lifetime of an unidentified 'alkali stabilized form of  $\text{O}_2^-$ ' absorbing at 240 m $\mu$  and described by Czapski & Dorfman (1964). Their data are replotted in Fig. 3. We conclude that their species is identical with ours.

### DISCUSSION

*Identity of the oxygen radical.* It is clear that the ESR signal described, which may be produced either by the enzyme system or by the inorganic system, is due to an oxygen free-radical species that appears identical with the unidentified 'alkali stabilized form of  $\text{O}_2^-$ ' of Czapski & Dorfman (1964).

One-electron reduction of oxygen at pH values above 4.45, which is the pK (Sehested, Rasmussen & Fricke, 1968) for the dissociation:



would be expected to yield the superoxide ion,  $\text{O}_2^-$ , and a number of ESR studies of this species have been carried out (Bennett, Ingram, Symons, George & Griffith, 1955; Känzig & Cohen, 1959; Lunsford & Jayne, 1966; Ichikawa *et al.* 1966). Although our parameters are in good agreement with those of, e.g., Ichikawa *et al.* (1966), very large effects of the surrounding matrix on the observed  $g$  values are expected for this species (Känzig & Cohen, 1959; Lunsford & Jayne, 1966; Atkins & Symons, 1967). Hence, although our parameters are fully consistent with our oxygen radical being  $\text{O}_2^-$  (Fig. 4), we do not consider that  $g$  values alone can provide rigorous identification. Further, Czapski & Dorfman (1964) in their pulsed-radiolysis studies on oxygenated water concluded that  $\text{O}_2^-$  and their 'alkali stabilized' species were distinct from one another. They found also that  $\text{O}_2^-$  is unstable, disappearing with a second-order rate constant of  $1.7 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$  at 25°. It is perhaps dubious whether such an unstable species could ever be trapped by our present rapid-freezing technique. Further investigations were therefore necessary to identify our species fully.

Studies with the inorganic system enabled production of our radical to be compared with the work

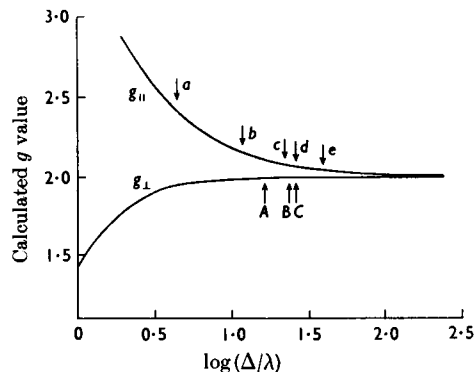
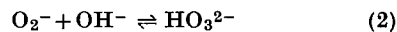


Fig. 4.  $g$  values for  $\text{O}_2^-$ . The curves represent theoretical plots of  $g_{\parallel}$  and  $g_{\perp}$  against the parameter  $\log(\Delta/\lambda)$ , calculated by a modification of the method described by Känzig & Cohen (1959) and taking  $E/\lambda = 350$  (Lunsford & Jayne, 1966).  $\log(\Delta/\lambda)$  is regarded as a function of the environment of the radical. The arrows indicate the  $g_{\parallel}$  values observed for  $\text{O}_2^-$  by various workers under differing experimental conditions. In every case the corresponding observed  $g_{\perp}$  values agree with those that would be predicted for the lower curve, within experimental error. *a*,  $\text{O}_2^-$  in irradiated KCl (Känzig & Cohen, 1959); *b*, in  $\text{Na}_2\text{O}_2$  (Bennett *et al.* 1955); *c*, in irradiated urea- $\text{H}_2\text{O}_2$  (Ichikawa *et al.* 1966); *d*, on MgO (Lunsford & Jayne, 1966); *e*, on ZnO (Lunsford & Jayne, 1966); *A*, from  $\text{H}_2\text{O}_2$ - $\text{IO}_4^-$ , pH 13 (Fig. 1c); *B*, from xanthine- $\text{O}_2$ -xanthine oxidase, pH 10 (Fig. 1a); *C*, from  $\text{H}_2\text{O}_2$ - $\text{IO}_4^-$ , pH 10 (Fig. 1b).

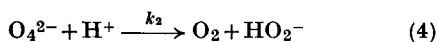
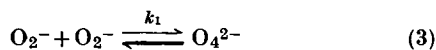
by Czapski & Dorfman (1964) on oxygen radicals and related species over a wider range of conditions than would have been possible with the enzyme system. These workers used only optical techniques, which are of course, unlike ESR, unable to distinguish between free radicals and other species. They detected a number of species derived from oxygen. However, only one of these, their 'alkali stabilized form of  $\text{O}_2^-$ ', could possibly be identical with our oxygen free radical species. Our conclusion that 'alkali stabilized form of  $\text{O}_2^-$ ' is a free radical makes it worth while to re-examine the question of its identity. The number of possible structures is of course very limited. First, three of the species Czapski & Dorfman (1964) propose,  $\text{O}_2^{2-}$ ,  $\text{O}_4^{2-}$  and  $\text{HO}_5^{3-}$ , would all be diamagnetic and so may be dismissed from further consideration. Another possibility that they mention is  $\text{HO}_3^{2-}$ , presumably formed by:



This would be paramagnetic and eqn. (2) could explain the pH data, if  $\text{HO}_3^{2-}$  is presumed to be stable. The change in  $g$  values with increasing pH would be explained by postulating that  $\text{O}_2^-$  and  $\text{HO}_3^{2-}$  are in rapid equilibrium so that only a single composite

signal is observed. However, this explanation can be rejected since we still obtained the same signal, rather than two separate signals, on cooling to 20°K. (We found, however, a small further increase in  $g_{\perp}$  at the helium temperature.) Further evidence against  $\text{HO}_3^{2-}$  was provided by the absence of proton splitting. In additional experiments, the radical was produced enzymically in  $\text{D}_2\text{O}$  in place of  $\text{H}_2\text{O}$ . The line width decreased only from 15 to 12 gauss, whereas  $\text{HO}_2$  shows a proton splitting of 11 gauss (Ichikawa *et al.* 1966). Finally, the axial symmetry shown by our species is also probably inconsistent with  $\text{HO}_3^{2-}$ .

We therefore prefer another explanation of the alkaline stability of the species (this being in fact only slightly modified from one put forward by Czapski & Dorfman, 1964). We propose that  $\text{O}_2^-$  is in rapid equilibrium with a small amount of a dimer and that radical disproportionation occurs via this dimer by a process that is proton-catalysed:



This explanation would appear to fit all the observations. Proton attack on the dimer would facilitate the intramolecular electron transfer required for disproportionation (eqn. 4). At neutral pH values  $k_2[\text{H}^+]$  is assumed to be large enough to make  $k_1$  rate-limiting in the decomposition, explaining the rapid second-order decay of  $\text{O}_2^-$  found in pulse-radiolysis work (Czapski & Dorfman, 1964). In alkaline solution  $k_2[\text{H}^+]$  would become rate-limiting, so explaining the rapid increase in stability with increasing pH in this region. According to this explanation, owing to the equilibrium in eqn. (3) favouring dissociation of the dimer, the species detected both optically and by ESR would of course be the parent species,  $\text{O}_2^-$ . In agreement with this, Czapski & Dorfman (1964) could not distinguish  $\text{O}_2^-$  and the 'alkali stabilized form of  $\text{O}_2^-$ ' optically and differentiated between them purely on kinetic grounds. Shifts in  $g$  values could be explained by matrix effects (Fig. 4). Unfortunately, it is not possible to predict the optical spectrum from the  $g$  values, as the constant  $\lambda$  is not known for  $\text{O}_2^-$  (Känzig & Cohen, 1959).

*Significance of  $\text{O}_2^-$  in the enzymic reaction.* Integration of the ESR signals showed that quite substantial concentrations of  $\text{O}_2^-$  could be attained relative to the starting oxygen concentration. Since the ion has only limited stability at pH10 it is clear that one-electron reduction to  $\text{O}_2^-$  plays a major role in oxygen utilization by the xanthine oxidase system under these conditions. It is to be noted that, instead of the characteristic  $\text{O}_2^-$  signal at pH10, a weaker symmetrical radical signal is seen

at pH 8. This is presumably due to the FADH semi-quinone (Bray, Malmström & Vänngård, 1959; Palmer *et al.* 1964). Although the  $\text{O}_2^-$  and FADH signals may readily be distinguished from one another by the asymmetry of the main  $g_{\perp}$  line of the former and by the presence of its  $g_{\parallel}$  bump, the detection of small amounts of one species in the presence of larger amounts of the other would not be easy. It seems likely that in earlier work (Palmer *et al.* 1964) the oxygen radical has to some extent been mistaken, particularly at high pH values, for FADH with possibly a consequent exaggeration of the role of the latter species in the mechanism of action of xanthine oxidase.

It is suggested that the present technique should serve as a clear-cut diagnostic test for one-electron reduction of oxygen in those enzyme reactions that can be studied at pH10. With xanthine oxidase, evidence of a less direct nature had already been obtained by others indicating oxygen radical involvement (Stauff, Schmidknecht & Hartmann, 1963; Totter, de Dugros & Riviero, 1960; Handler, Rajagopalan & Aleman, 1964). It is noteworthy that Totter, Medina & Scoseria (1960) found pH effects similar to ours in luminescence studies on the enzyme. However, our conclusion that the  $\text{O}_2^-$ -free radical is stabilized by alkaline media, rather than by being bound to the enzyme molecule in some way, appears to be an entirely new one. Although the present work gives no indication as to what groupings in the xanthine oxidase molecule produce the  $\text{O}_2^-$  radicals, iron seems to be the most likely site. Bray *et al.* (1964) obtained evidence that reoxidation of the enzyme by oxygen may occur via iron, and Handler *et al.* (1964) found that only non-haem iron-containing flavoproteins give any evidence of radical production in the test systems that they employed.

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